Nosema ceranae has been infecting honey bees Apis mellifera in Italy since at least 1993

NOTES AND COMMENTS

Nosema ceranae has been infecting honey bees *Apis mellifera* in Italy since at least 1993.

Ezio Ferroglio[1*], Stefania Zanet[1], Nancy Peraldo[2] , Elisa Tachis[1], Anna Trisciuoglio[1], Daniela Laurino[2], Marco Porporato[2].

[1]Dipartimento di Produzioni Animali, Epidemiologia ed Ecologia, Università di Torino, via L. Da Vinci, 44 10095 Grugliasco (TO) Italy.
[2] Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Via L. Da Vinci, 44 10095 Grugliasco (TO) Italy.

Received 16 May 2012, accepted subject to revision 14 August 2012, accepted for publication 12 September 2012.

***Corresponding author:** Email: ezio.ferroglio@unito.it

Key words: *Nosema ceranae,* honey bees, Italy, PCR

Short title: *Nosema ceranae* in Italy

Summary

Nosema ceranae is a microsporidian parasite regarded as one of the emerging pathogens affecting honey bees worldwide. Originally found in the Asian honey bee *A. cerana,* its presence in Europe can be dated back to 1998. A temporal sequence of N=6 samples from Northwestern Italy from 1993 to 2010 was analyzed by PCR for the presence of *N. ceranae* and *N. apis. N. ceranae* was detected in 4 samples (1 from 1993, 1 from 1998, 2 from 2010), *N. apis* was detected in 2 samples from 1993. The resulting *N. ceranae* sequence fragments showed 100% homology with *N. ceranae* Genebank-deposited sequences. Our data provide evidence that *N. ceranae* has been present in honey bees from NW Italy since at least 1993, and that the pathogen was introduced in honey bees in Europe well before its first report in 2005.

Nosema ceranae, originally described in the Asian honey bee *Apis cerana*, is considered an emerging infectious disease in Europe and in other countries where *A.*

mellifera is present (Fries, 2010). *Nosema ceranae* has spread rapidly across the world (Klee *et al.,* 2007) and it is considered to be one of the risk factors related to "Colony Collapse Disorder" (CCD) (Williams *et al*., 2008; Paxton, 2010). We analyzed honey bees for the presence of *Nosema ceranae* in historic samples available at the bee archive of the Faculty of Agronomy, University of Turin, Italy, as well as in newly collected samples from the same area in Northwestern Italy; the available samples dated back to 1993. Adult bees were collected from 3 colonies in 1993 (San Raffaele Cimena, 45° 9′ 0″ N, 7° 51′ 0″ E) and 1 colony in 1998 (Monteu Roero, 44° 46′ 49.08″ N, 7° 56′ 15″ E). Honey bees were also collected in 2010 from 2 colonies (Valfenera, 44° 54′ 0″ N, 7° 58′ 0″ E, and Grugliasco, 45° 4′ 0″ N, 7° 35′ 0″ E) (Table 1). Bees from each colony were stored in vials (one colony per vial) and frozen at -20°C until further processing. Abdomens of 10 adult honey bees from each of the sampled colonies were macerated in 10 ml of saline solution (0.9% NaCl). Total genomic DNA was extracted by collecting 400 µl from each sample, and centrifuging it for 5 minutes at 16,000 *g*. Spores were further washed in Germination Buffer (De Graaf *et al*., 1993). Each sample was centrifuged at 16,000 *g* for 5 minutes, the pellet washed with 200 µl saline solution (0.9% NaCl) and centrifuged again at 16,000 *g* for 5 minutes. Total genomic DNA was extracted from the pellet using Gen Elute™ Mammalian Genomic DNA extraction kit (Sigma-Aldrich; St. Louis, MO, USA) following the manufacturer's instructions. Negative controls (purified water) were processed in parallel at all steps from extraction to amplification, to detect possible contamination, and all tested negative at PCR. All samples were double-tested by PCR according to the method described by Martin-Hernandez *et al.* (2007) in order to differentiate *N. apis* and *N. ceranae*. Resulting amplicons were separated on a 2% agarose gel, stained with GelRed Nucleic Acid Gel Stain in Water (VWR Int. Milano, Italy) with Pb 100 Molecular Weight Marker (Sigma-Aldrich; St. Louis, MO, USA). *N. ceranae* positive samples were purified using the commercial kit NucleoSpin Extract II (Macherey-Nagel Int., Düren, Germany) following the manufacturer's instructions for DNA gel extraction. Purified amplicons were ligated into the pDrive cloning vector and transformed into Qiagen EZ competent *E. coli* cells, as specified by the manufacturer (Qiagen PCR Cloning Kit Plus, Qiagen Int.; Milan, Italy). Cloned plasmid DNA was isolated using a QIAprep Spin Miniprep

Kit according to the QIAprep Miniprep Handbook (Qiagen Int.; Milan, Italy), dehydrated and sent to BMR Genomics (Padua, Italy) for sequencing.

Two of three samples from 1993 were positive for *N. apis* and one was also positive for *N. ceranae* by PCR (Table 1). The three samples from 1998 and 2010 were all positive for *N. ceranae* and negative for *N. apis* by PCR (Table 1). The *N. ceranae* 16 r-RNA gene sequence was confirmed from all three *N. ceranae*- specific PCR products from 2010 and 1998, and in the one sample from 1993 harbouring both *Nosema* species (PCR amplicons specific fo *N. apis* and *N. ceranae* respectively were obtained from this sample, and sequenced separately). All samples positive for *N. ceranae* were cloned and sequenced to confirm PCR results. Sequences obtained were compared to the ones available in online-databases. All showed 100% homology with already available *N. ceranae* sequences. All four sequences were submitted to GeneBank (www.ncbi.nlm.nih.gov) and assigned Accession Nos. HM859896 to HM859899. All sequences obtained in the present study were identical apart from one single nucleotide at position 34; samples N076-10 (HM859899) and N099-93 (HM859897) had a guanidine, while samples N050-98 (HM859896) and N063-10 (HM859898) had an adenine.

Nosema ceranae was recently reported to infect *A. mellifera* in Europe (Higes *et al.,* 2006) and across the world (Klee *et al.,* 2007). Several authors claimed that this emerging pathogen is involved in the honey bee CCD syndrome based on both field and experimental data (Williams *et al*., 2008; Alaux *et al.,* 2011).These reports seem to indicate that *N. ceranae* has been introduced in Northern Europe at least since 1998 (Paxton *et al*., 2007). However there is no other evidence of *N. ceranae*'s presence in honey bees in Europe before 2005 (Fries, 2010). Our data show that *N. ceranae* has been present in NW Italy since 1993. The finding of *N. ceranae* spores from adult bees collected and stored in 1993 suggests that this pathogen was present in Europe well before its first description in 2005.

Apparently there are no differences in the *N. ceranae* sequences from bees collected in 1993, in 1998 and in 2010, similar to the findings of Williams *et al.* (2008) who found that only one *N. ceranae* haplotype is present in North American honey bee colonies. This could be due to the small sequence amplified by the PCR protocol we used,

but also to the short period from its introduction in the area or, more likely, to a founder effect due to a single source.

In spite of the few available samples, we suggest *N. apis* may be displaced by *N. ceranae*, but any conclusion could be made only if more historic samples become available. *Nosema* diagnosis has been undertaken traditionally by optical microscopy. We lack data on the introduction and spread in the 1990s of *N. ceranae*, which has possibly been misdiagnosed as *N. apis* for decades. The finding of *N. ceranae* in 1993, 5 years before its first report in Fennoscandia (Paxton *et al.,* 2007), could support the hypothesis that *N. ceranae* was first introduced in Southern Europe and lately spread across the continent and, by bees and bee-product trade, to other parts of the world.

Acknowledgements

This research was partly funded by a research grant (Messa a punto di metodi diagnostici innovativi e loro applicazione al controllo della peste americana e nosemiasi) of the Regione Piemonte, Assessorato all'Agricoltura. We also want to thank two anonymous referees for they helpful comments on the manuscript.

References

ALAUX, C; FOLSCHWEILLER, M; MCDONNELL, C; BESLAY, D; COUSIN, M; DUSSAUBAT, C; BRUNET, J L; LE CONTE, Y (2011) Pathological effects of the microsporidium *Nosema ceranae* on honey bee queen physiology (*Apis mellifera*). *Journal of Invertebrate Pathology* 106(3): 380–385. http://dx.doi.org/10.1016/j.jip.2010.12.005

DE GRAAF, D C; MASSCHELEIN, G; VANDERGEYNST, F; DE BRAVANDER, H F; JACOBS, F J (1993) In vitro germination of *Nosema apis* (Microspora: Nosematidae) spores and its effects on their αα-trehalose/D-glucose ratio. *Journal of Invertebrate Pathology* 62(3): 220–225. http://dx.doi.org/10.1006/jipa.1993.1103

FRIES, I (2010) *Nosema ceranae* in European honey bees (*Apis mellifera*). *Journal of Invertebrate Pathology* 103: S73–S79. http://dx.doi.org/10.1016/j.jip.2009.06.017

HIGES, M; MARTIN, R; MEANA, A (2006) *Nosema ceranae*, a new microsporidian parasite in honey bees in Europe. *Journal of Invertebrate Pathology* 92: 93-95. http://dx.doi.org/10.1016/j.jip.2006.02.005

KLEE, J; BESANA, A M; GENERSCH, E; GISDER, S; NANETTI, A; TAM, D Q; CHINH, T X; PUERTA, F; RUZ, J M; KRYGER, P; MESSAGE, D; HATJINA, F; KORPELA, S; FRIES, I; PAXTON, R J (2007) Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology* 96: 1-10. http://dx.doi.org/10.1016/j.jip.2007.02.014

MARTÍN-HERNÁNDEZ, R; MEANA, A; PRIETO, L; MARTINEZ SALVADOR, A; GARRIDO-BAILON, E; HIGES, M (2007) Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Applied Environmental Microbiology* 73(20): 6331–6338. http://dx.doi.org/10.1128/AEM.00270-07

PAXTON, R J; KLEE, J; KORPELAB, S; FRIES, I (2007) *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. *Apidologie* 38(6): 558–565. http://dx.doi.org/10.1051/apido:2007037

PAXTON, R J (2010) Does infection by *Nosema ceranae* cause "Colony Collapse Disorder" in honey bees (*Apis mellifera*)? *Journal of Apicultural Research* 49(1): 80-84. http://dx.doi.org/10.3896/IBRA.1.49.1.11

WILLIAMS, G R; SHAFER, A B A; ROGERS, R E L; SHUTLER, D; STEWART, D T (2008) First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (Apis mellifera), in Canada and central USA. *Journal of Invertebrate Pathology* 97(2): 189-192. http://dx.doi.org/10.1016/j.jip.2007.08.005

Year	**Sample ID**	**Origin**	***N. apis* PCR** (Martin-Hernandez et al., 2007)	***N. ceranae* PCR** (Martin-Hernandez et al., 2007)	**GenBank Accession no.** *N. ceranae* 16S ribosomal RNA gene partial sequence
1993	N097-93	San Raffaele Cimena	negative	negative	--
	N098-93	San Raffaele Cimena	positive	negative	--
	N099-93	San Raffaele Cimena	positive	positive	HM859897
1998	N050-98	Monteu Roero	negative	positive	HM859896
2010	N063-10	Grugliasco	negative	positive	HM859898
	N076-10	Valfenera	negative	positive	HM859899

Table 1. Adult honey bee samples collected and tested by PCR for the presence of *Nosema ceranae* and *Nosema apis* DNA from North West Italy.